ELSEVIER

Biochimica et Biophysica Acta 1405 (1998) 85–98

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

Nucleation and elongation of actin filaments in the presence of high speed supernate from neutrophil lysates: modulating effects of Ca^{2+} and phosphatidylinositol-4,5-bisphosphate

Mark J. DiNubile *

Division of Infectious Diseases, Department of Medicine, Cooper Health System, UMDNJ/Robert Wood Johnson Medical School, Camden, NJ, USA

Received 9 April 1998; revised 5 August 1998; accepted 20 August 1998

Abstract

Cell motility depends on the rapid growth of cortical actin filaments whose barbed ends are capped in the resting cell. High speed supernates (HSS) of dilute neutrophil lysates contain actin monomers and/or oligomers that can be induced to polymerize by certain stimuli. We questioned whether some of the actin remaining in the supernate after high speed centrifugation exists as occult nucleation sites which can elongate when uncapped. Phosphatidylinositol-4,5-bisphosphate (PIP_2) may play a critical role as an intracellular messenger in cytoskeletal rearrangement after stimulation by removing cappers from barbed filament ends. The experiments reported here examine the separate and interactive effects of PIP_2 micelles and micromolar [Ca^{2+}] on the rates of nucleation and elongation of pyrenyl-G-actin in the presence of HSS. HSS slowed the nucleation and elongation rates of gel-filtered pyrenyl-G-actin polymerized at submicromolar [Ca^{2+}]. Under these conditions, PIP_2 only slightly increased the number of nucleation sites, but delayed the slowing of the elongation rate in the presence of HSS. Nucleating activity in HSS could be induced by the addition of micromolar [Ca^{2+}] and totally abolished by immunoprecipitation of gelsolin from HSS; incubation of HSS with PIP_2 at micromolar [Ca^{2+}] slightly decreased the number of calcium-induced nucleation sites in the supernate. Incubation of HSS with PIP_2 before the addition of calcium led to a greater reduction in Ca^{2+}-inducible nucleation sites. HSS possessed more nucleation sites after simultaneous exposure to PIP_2 and Ca^{2+}, followed by chelation of Ca^{2+} with EGTA, than HSS preincubated at micromolar [Ca^{2+}] without PIP_2. At submicromolar [Ca^{2+}], PIP_2 only generated a few barbed end nucleation sites in the HSS, but lessened the gradual slowing of elongation seen with HSS in the absence of PIP_2, presumably by preventing capping by capping protein-β_2 in the supernate. Pointed end nucleating sites in HSS, attributable to gelsolin, could be created by adding micromolar [Ca^{2+}]. The preincubation of HSS with PIP_2 in the absence of micromolar [Ca^{2+}] decreased the number of Ca^{2+}-inducible nucleation sites in the HSS. Under conditions mimicking the sequential rise and fall of cytosolic [Ca^{2+}] after stimulation, PIP_2 accelerated actin polymerization despite the inhibitory action of HSS by maintaining Ca^{2+}-activated nucleation sites. These observations suggest that a possible role for PIP_2 in modulating cytoskeletal growth in vivo may be to regulate nucleation sites activated by sequential changes in cytosolic [Ca^{2+}].

Keywords: Phosphatidylinositol-4,5-bisphosphate; Calcium; Capping protein-β_2; Actin

* Room 274, Education and Research Building, 401 Haddon Avenue, Camden, NJ 08103, USA. Fax: +1 (609) 757-7803; E-mail: dinubile@umdnj.edu.

0167-4889/98/$ – see front matter
PII: S0167-4889(98)00108-6

1. Introduction

Despite buffer conditions in the cell favorable for the polymerization of actin, nearly equivalent amounts of cytoplasmic actin are in the monomeric globular (G) and filamentous (F) forms [1]. However, phagocytes can quickly double the amount of F-actin upon exposure to chemoattractants [2,3]. Since spontaneous nucleation at the concentrations of G-actin present in the cytosol occurs too slowly to explain the observed rapid rate of polymerization, the kinetics of cytoskeletal rearrangements after cell stimulation cannot be explained simply by mobilization of G-actin from sequestered sites. Accordingly, agonists must also induce the formation of new nucleation sites for filament growth [4].

Nucleation sites might be generated by activating nucleating proteins or releasing occult nucleation sites in the cytoplasm. Preformed nucleation sites can be exposed by the uncapping of pre-existent actin filaments with or without severing. A variant of this possibility involves increasing the availability of actin oligomers, by uncapping and/or unbundling, to act as seeds for filament elongation [5]. Cutting of actin filaments without capping is another potential mechanism by which the number of growing ends can be rapidly augmented.

Data in platelets suggest that new nucleation sites may be created by the sequential action of calcium and PIP_2, first to activate gelsolin to cut pre-existent actin filaments and cap the newly formed barbed ends and then to uncap both the original and just created ends [4,6,7]. Gelsolin as well as the resting capper (presumed to be capping protein-β_2) are simultaneously removed from the barbed ends by the formation of PIP_2 and the lowering of cytosolic calcium [4,7].

In the neutrophil, chemoattractants rapidly lead to an approximately two-fold increase in both the number of filaments and the concentration of F-actin [3]. The increase in the F-actin content after exposure of streptolysin-O permeabilized neutrophils to GTPγS depends on the availability of free barbed ends [8]. High speed supernates (HSS) of dilute neutrophil lysates contain actin that can be induced to polymerize under certain conditions [9]; it is unclear whether some of the actin remaining in the supernate after lysis, dilution and high speed centrifugation exists as oligomers which are capable of nucleating polymerization when uncapped. The addition of cdc42 charged with GTPγS to HSS, but not GTPγS alone, results in a quick and substantial increase in the amount of sedimentable actin at submicromolar [Ca^{2+}] [9] HSS exposed to micromolar [Ca^{2+}] causes exogenous actin to polymerize without a lag phase, presumably through gelsolin-mediated nucleation and pointed end elongation.

We decided to study the kinetic behavior of HSS as a first approximation for the events that may occur in the cytosol of neutrophils after stimulation by chemoattractants. This surrogate system allowed us to test earlier observations made on purified proteins in the more complex milieu of multiple interacting cytosolic components, while bypassing the barrier of the plasma membrane [9]. Since both Ca^{2+} and PIP_2 are widely accepted as key second messengers in signal transduction which act directly on actin binding proteins to promote rapid polymerization [4,10–14], we focused on the individual and interactive effects of these two mediators on the rates of actin nucleation and subsequent elongation. Our observations revealed that when preincubated together at submicromolar [Ca^{2+}], PIP_2 reduced the number of Ca^{2+}-inducible nucleation sites in HSS as expected, but paradoxically maintained Ca^{2+}-induced nucleation sites after [Ca^{2+}] had been lowered.

2. Materials and methods

2.1. Procurement of rabbit neutrophils

Neutrophils were obtained from rabbit peritoneal exudates, as described by Sullivan and Zigmond [15] except that the animals were sedated with 0.02 mg/kg of acepromazine maleate (Germenta, Kansas City, MO) 20 min prior to handling.

2.2. Preparation of neutrophil lysates

Neutrophils were resuspended at 2×10^8 cells/ml in a lysis buffer containing 10 mM HEPES, 2 mM $MgCl_2$, 150 mM KCl, 5 mM EGTA, 2 mM potassium phosphate, 10 mM β-glycerol phosphate, 0.2 mM dithiothreitol (DTT), 5 mM ATP, brought to pH 7.2 with NaOH. Just before lysis, protease inhibitors

with final concentrations of 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin, 1 μg/ml benzamide, 10 μg/ml aprotinin and 10 μg/ml TAME-HCl were added. Cells were then incubated on ice in a Parr bomb at 350 psi of N_2 for 15 min before pressure was released [16]. The extracts created by nitrogen cavitation were immediately spun for 20 min at $100\,000 \times g$ (80 000 rpm in the 100.3 rotor) in a Beckman TL-100 tabletop ultracentrifuge at 4°C. This 'HSS' was stored at −80°C. Typically, HSS contained 2 mg protein/10^8 lysed cells. For some experiments, neutrophils were lysed on ice in the lysis buffer previously described at a concentration of 3×10^8 by sonication using a Dynatech Sonic Dismembrator (model 150) for 1 s every 4–5 s for three cycles. The sonicated extracts were spun at 14 000 rpm for 5 min in an Eppendorf 5412 centrifuge at 4°C and then this supernate was spun again in a Beckman TL-100 ultracentrifuge as detailed above to make HSS.

2.3. Preparation of pyrenyl-G-actin

Actin was purified from acetone powder extracted from the skeletal muscle of New Zealand white rabbits by the method of Spudich and Watt [17]. Pyrenylactin was prepared from rabbit skeletal muscle actin as described by Murray et al. [18]. The pyrenyl-G-actin was applied to a Sephacryl S-200 (Pharmacia, Piscataway, NJ) gel filtration column and then maintained in a *Storage buffer*, containing 5 mM triethanolamine, 0.3 mM $CaCl_2$, 0.68 mM ATP, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.02% NaN_3, pH 7.5, at 4°C. The critical actin monomer concentration varied between 0.1 and 0.2 μM for different preparations.

2.4. Preparation of gelsolin

Plasma gelsolin was isolated from rabbit serum (Life Technologies, Gaithersburg, MD) as described by Cooper [19].

2.5. Sedimentation assay and quantitative Western blotting

Pyrenyl-G-actin at a concentration of 10 μM was copolymerized with 3 μl HSS from neutrophils lysed at 2×10^8 cells/ml by nitrogen cavitation in 100 μl of either Ca- or EGTA-buffer for 3 h at 37°C. Specimens were then spun at $100\,000 \times g$ for 15 min at room temperature in a Beckman Airfuge ultracentrifuge. The supernatants were discarded and the pellets washed twice with 100 μl of the corresponding buffer. The pellets were then resuspended in 30 μl gel sample buffer and applied to SDS-10% polyacrylamide minigels. After transfer to Immobilon-P membranes (Millipore, Bedford, MA), Western blotting was used to quantitate the amount of gelsolin and capping protein-β_2 in the pellets. Primary antibodies were monoclonal antibodies against human plasma gelsolin (GS-234; Sigma, St. Louis, MO) and against capping protein-β_2 (mAb 3F2.3; University of Iowa Developmental Studies Hybridoma Bank). Peroxidase-conjugated secondary antibodies (Sigma) were detected by enhanced chemiluminescence (ECL). ECL molecular weight markers were purchased from Amersham, Arlington Heights, IL.

2.6. Preparation of phosphatidylinositol-4,5-bisphosphate and other phospholipids

Phosphatidylinositol-4,5-bisphosphate (PIP_2), phosphatidylinositol-4-monophosphate (PIP), and phosphatidylinositol (PI), purchased from Sigma (St. Louis, MO), were dissolved in deionized–distilled water at 1 mg/ml and immediately sonicated on ice at maximum power for 3–4 min using an Ultrasonic Cell Disrupter. Working aliquots were frozen using liquid nitrogen and stored at −60°C. Immediately before use, PIP_2 was thawed and sonicated on ice at maximum power for 1 min.

2.7. Actin polymerization assay

Polymerization of pyrenyl-G-actin was determined from the change in pyrenylactin fluorescence (excitation at 370 nm; emission at 410 nm) in a Perkin-Elmer LS5 fluorimeter. EGTA- and Ca-buffers used for the incubations and assays contained 25 mM Tris-HCl, 138 mM KCl, 2 mM $MgCl_2$, and 1 mM ATP, pH 7.4 with either 1 mM EGTA or 200 μM calcium, respectively. For selected experiments where noted, magnesium was omitted from the EGTA-buffer.

Fluorescence readings were zeroed by subtracting

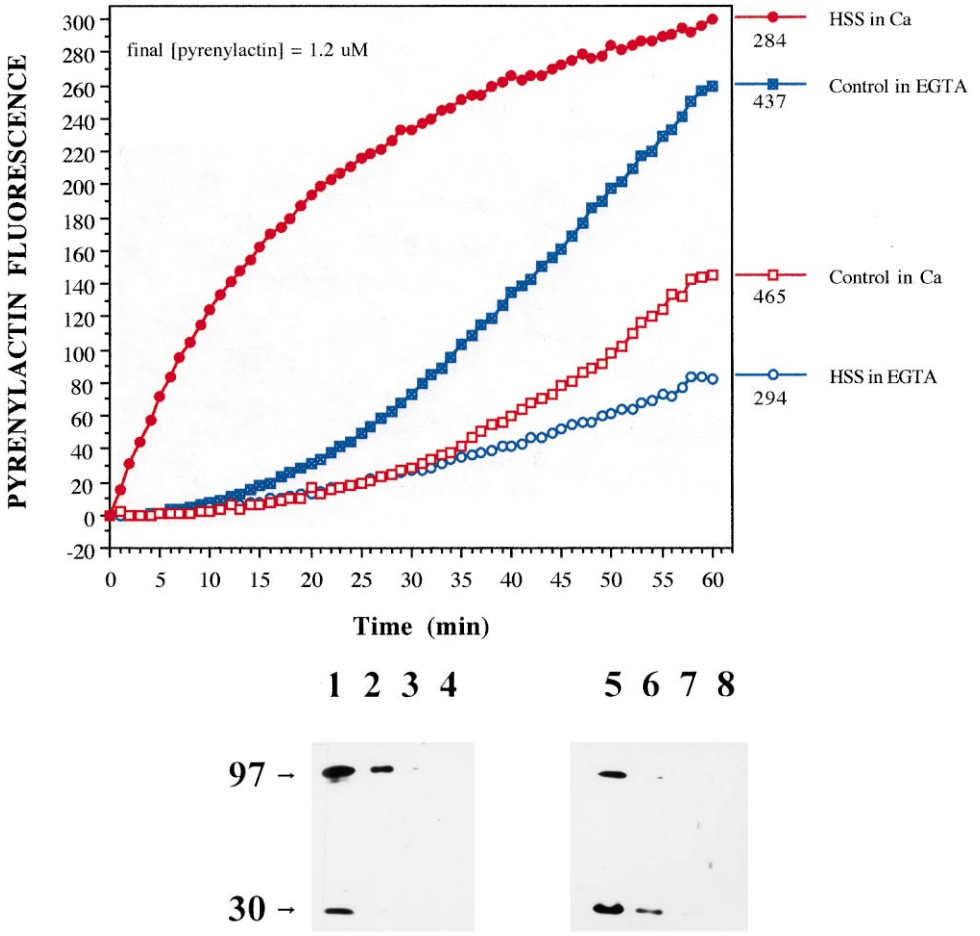


Fig. 1. (A) High speed supernate (HSS) of dilute neutrophil lysates inhibited nucleation of pyrenyl-G-actin at submicromolar [Ca^{2+}], but nucleated actin polymerization at micromolar [Ca^{2+}]. HSS (5 μl) from neutrophils lysed by sonication at 3×10^8 cells/ml was incubated in polymerization buffer containing either 1 mM EGTA (EGTA-buffer) or 0.2 mM $CaCl_2$ (Ca-buffer) for 5 min at room temperature. Controls contained 5 μl of buffer instead of HSS. Samples were then brought to 1 ml with the corresponding buffer containing 1.2 μM pyrenyl-G-actin. Fluorescence was recorded at 1 min intervals for 60 min; steady-state fluorescence (shown under the legend to the right of the figure) was read after overnight incubation in the dark at room temperature. Fluorescence values were zeroed for each sample by subtracting the fluorescence value immediately after mixing each sample from all readings. Immunodepletion of gelsolin from HSS removed its nucleation activity in Ca-buffer. (B) More gelsolin pelleted with actin copolymerized with HSS at micromolar [Ca^{2+}], whereas more capping protein-β_2 pelleted with actin copolymerized with HSS at submicromolar [Ca^{2+}]. Pyrenyl-G-actin at a concentration of 10 μM was copolymerized with 3 μl HSS from neutrophils lysed at 2×10^8 cells/ml by nitrogen cavitation in 100 μl of either Ca- or EGTA-buffer for 3 h at 37°C. Specimens were then spun at $100\,000\times g$ for 15 min at room temperature. After washing, pellets were resuspended in 30 μl gel sample buffer, run on a SDS–10% polyacrylamide minigel and transferred to Immobilon-P membranes for simultaneous immunoblotting with monoclonal antibodies against human plasma gelsolin and against capping protein β_2. Lanes 1–4 contain serial two-fold dilutions of the actin pellets polymerized in Ca-buffer, starting with 8 μl; lanes 5–8 hold the equivalent samples polymerized in EGTA-buffer. These results suggest competition between gelsolin and capping protein β_2 for filament binding; the presence of micromolar [Ca^{2+}] favors gelsolin.

the fluorescence of pyrenyl-G-actin immediately after its addition to polymerization buffer under each individual condition. Steady state was assumed to have been reached after overnight incubation of samples at room temperature in the dark.

2.8. Immunoprecipitation of gelsolin from HSS

GammaBind Plus Sepharose beads (Pharmacia) were incubated overnight with phosphate-buffered saline (as a control) or monoclonal antibodies

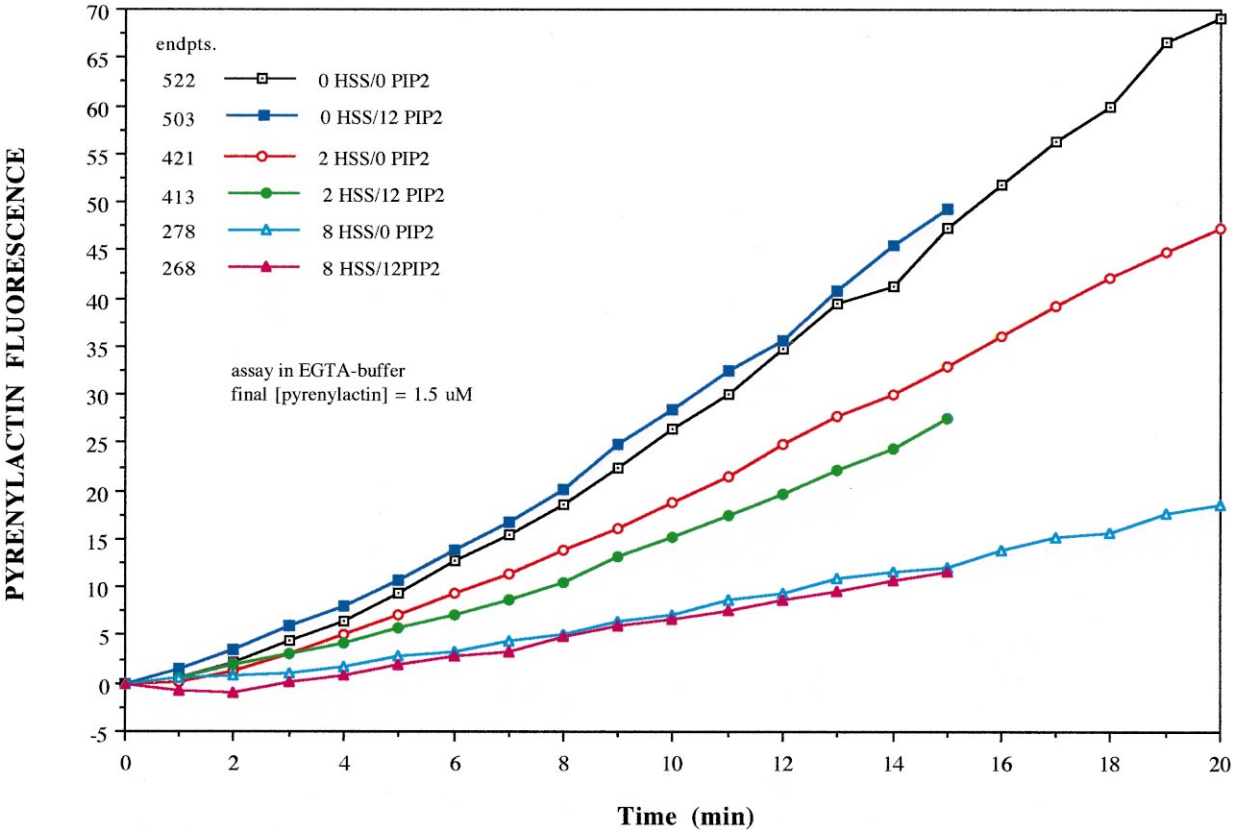


Fig. 2. HSS slowed pyrenyl-G-actin polymerization in a dose-dependent manner, which was unaffected by low concentrations of PIP_2. Different amounts of HSS from neutrophils lysed by nitrogen cavitation at 2×10^8 cells/ml were mixed with deionized–distilled water or 12 μM PIP_2 in 1 ml EGTA-buffer containing 1.5 μM pyrenyl-G-actin.

against human plasma gelsolin (GS-234; Sigma). After washing the beads with 1% milk and phosphate-buffered saline, HSS was applied to each set of beads for nearly 24 h. The next day, the beads were spun briefly at low speed and the supernate recovered for Western blotting and actin nucleation assays. This procedure generated gelsolin-depleted HSS.

3. Results

3.1. *Supernates slowed actin polymerization at submicromolar [Ca^{2+}], but nucleated filament growth in the presence of micromolar [Ca^{2+}]*

Actin, gelsolin and capping protein-β_2 could be demonstrated by Western blotting in the supernate from dilute neutrophil lysates after high speed centrifugation. These HSS prolonged the lag phase and slowed the subsequent elongation rate of exogenous pyrenyl-G-actin in polymerization buffer at submicromolar [Ca^{2+}], but nucleated polymerization at micromolar [Ca^{2+}] (Fig. 1A).

The fluorescence 'endpoints' after overnight incubation (shown in Fig. 1A) were nearly the same for corresponding samples in high or low [Ca^{2+}]. However, both samples containing HSS exhibited less fluorescence at steady state than samples without supernate, a finding consistent with the expected increase in the actin monomer concentration when almost all the barbed ends are capped by supernate. In addition to Ca^{2+}-activated gelsolin, this explanation requires the presence of a Ca^{2+}-independent capper in the HSS; we had previously demonstrated sufficient quantities of capping protein-β_2 in dilute HSS to cap all the barbed ends under the conditions used here [20].

To confirm that barbed filament ends were being capped by HSS regardless of the [Ca^{2+}], we studied

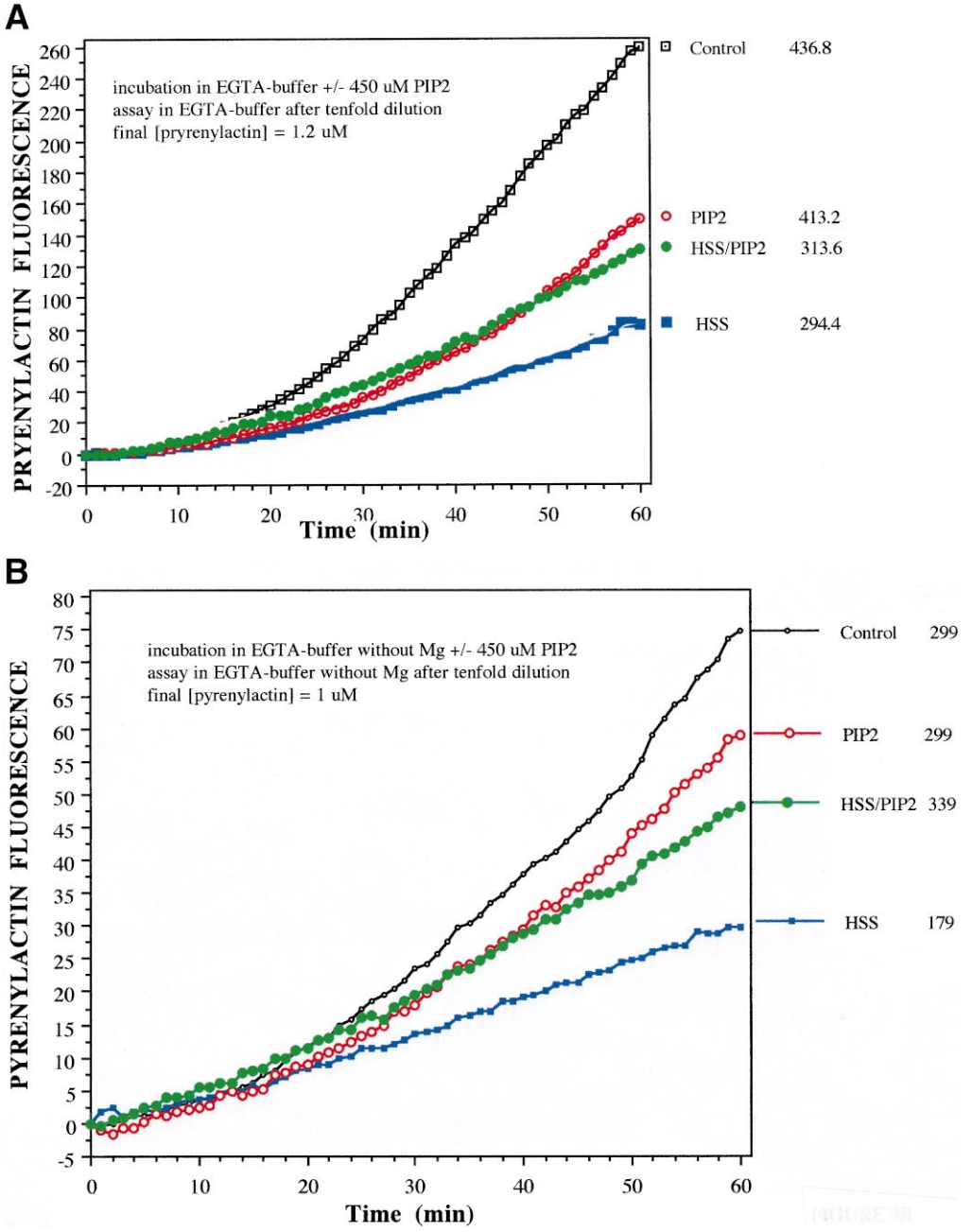


Fig. 3. (A) High concentrations of PIP_2 increased the pyrenyl-G-actin polymerization rate in the presence of HSS. A 5-μl amount of polymerization buffer or HSS from neutrophils lysed by sonication at 3×10^8 cells/ml was incubated with 45 μl deionized–distilled water or 450 μM PIP_2 for 5 min at room temperature in EGTA-buffer. Samples were then bought to 1 ml with EGTA-buffer containing 1.2 μM pyrenyl-G-actin. Steady-state fluorescence (shown next to the legend at the right of the figure) was read after overnight incubation in the dark at room temperature. (B) The absence of magnesium from the incubation and assay buffers did not substantially enhance the effects of PIP_2 on pyrenyl-G-actin polymerization caused by HSS. A 5-μl amount of EGTA-polymerization buffer or HSS from neutrophils lysed at 2×10^8 cells/ml by nitrogen cavitation was incubated with 45 μl deionized–distilled water or 450 μM PIP_2 in 100 μl EGTA-buffer without magnesium for 5 min at room temperature. Samples were then bought to 1 ml with EGTA-buffer without magnesium containing 1 μM pyrenyl-G-actin. Steady-state fluorescence (shown next to the legend at the right of the figure) was read after overnight incubation in the dark at room temperature.

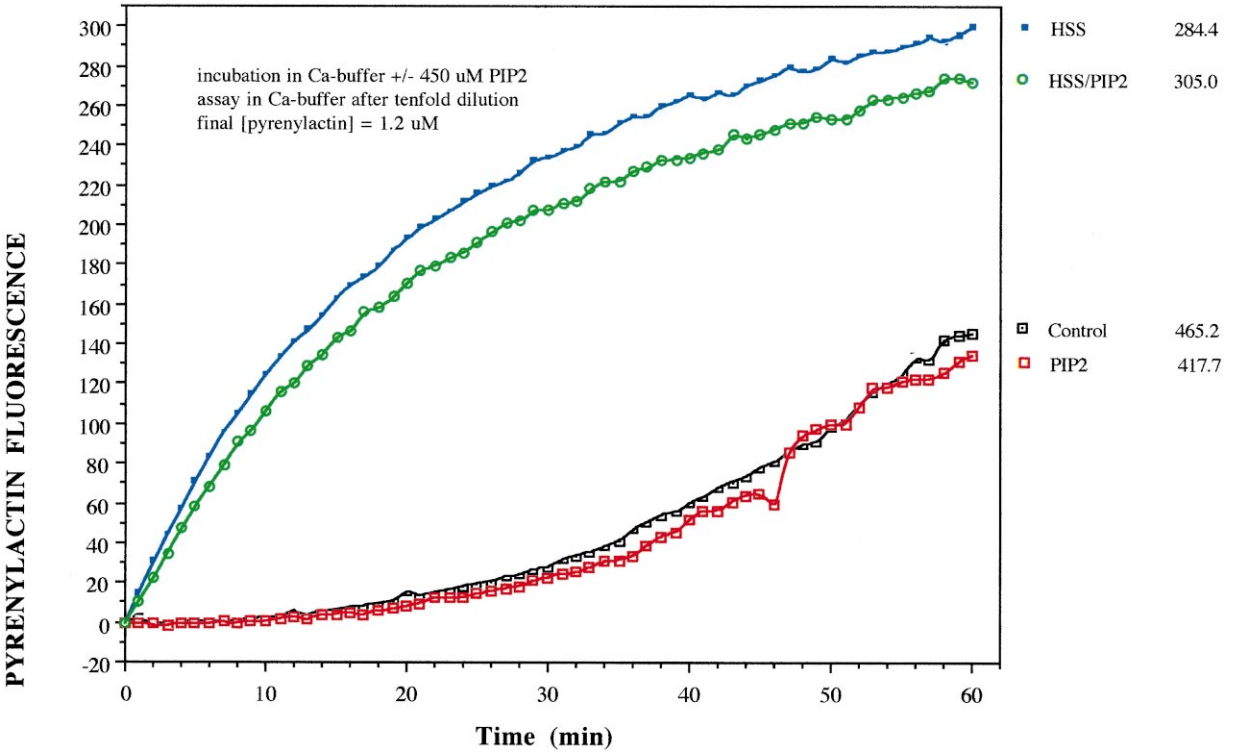


Fig. 4. PIP_2 had little effect on the polymerization rates of pyrenyl-G-actin polymerization in the presence or absence of HSS in buffer containing micromolar [Ca^{2+}]. A 5-μl amount of Ca-buffer or HSS from neutrophils lysed by sonication at 3×10^8 cells/ml was incubated with 45 μl deionized–distilled water or 450 μM PIP_2 for 5 min at room temperature in Ca-buffer. Samples were then bought to 1 ml with Ca-buffer containing 1.2 μM pyrenyl-G-actin. Steady-state fluorescence (shown next to the legend at the right of the figure) was read after overnight incubation in the dark at room temperature.

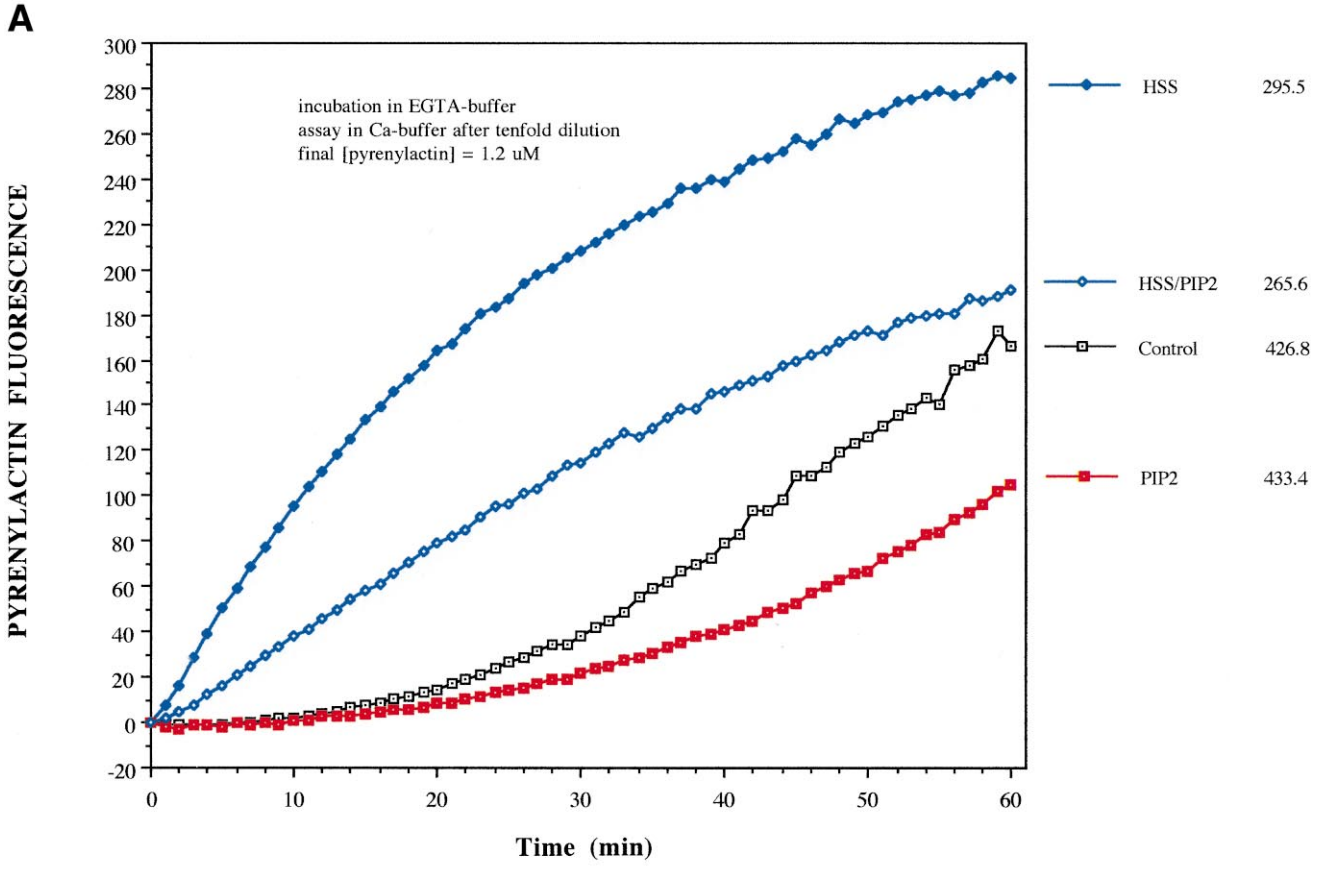
the effects of adding 0.5 μM cytochalasin B, a barbed end capper (and weak nucleator of pointed end growth) [19,21]. Cytochalasin B only slowed the polymerization rate in the absence of HSS. The fluorescence endpoints for samples containing HSS and/or cytochalasin B were reduced, implying that the majority of barbed ends were capped if either HSS or cytochalasin B were present. Although the kinetics of capping differed, the degree of capping at steady state was basically equivalent in the presence of HSS, with or without cytochalasin B, at micromolar and submicromolar [Ca^{2+}].

The activation of gelsolin in the HSS by Ca^{2+} would explain the observed increase in nucleation sites with HSS at micromolar [Ca^{2+}]. Incubating HSS with monoclonal antibodies against gelsolin substantially reduced the Ca^{2+}-inducible nucleation sites. Likewise, immunoprecipitation of gelsolin completely removed all nucleating activity from HSS. Approximately twice as much gelsolin pelleted with actin copolymerized with HSS in Ca-buffer as opposed to EGTA-buffer (Fig. 1B); the opposite results were seen with capping protein β_2, suggesting a competitive advantage for capping by gelsolin in the presence of micromolar [Ca^{2+}].

The inverse dependence of polymerization rate on the concentration of HSS in EGTA-buffer is depicted in Fig. 2. The decrement in steady-state fluorescence was likewise a function of the amount of HSS added to the sample.

3.2. PIP_2 only slightly increased the nucleation rate, but did inhibit the gradual slowing of the elongation rate in the presence of supernate at submicromolar [Ca^{2+}]

As seen in Fig. 2, 'low' concentrations of PIP_2 effective in inhibiting purified capping protein and gelsolin had no effect on actin polymerization in the presence or absence of HSS. However, high con-


A
PYRENYLACTIN FLUORESCENCE
incubation in EGTA-buffer
assay in Ca-buffer after tenfold dilution
final [pyrenylactin] = 1.2 uM
HSS 295.5
HSS/PIP2 265.6
Control 426.8
PIP2 433.4
Time (min)

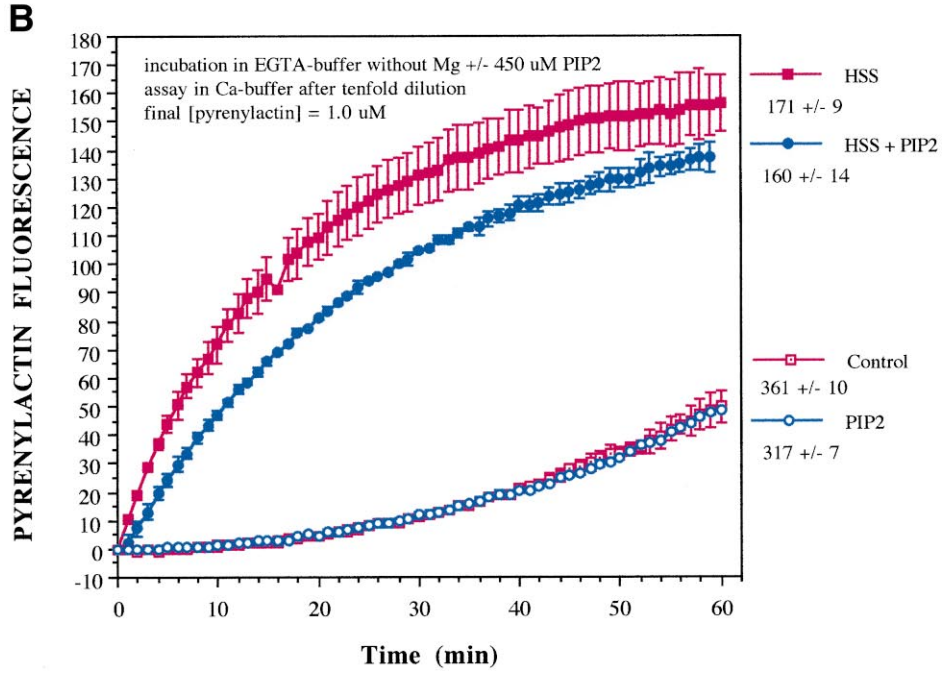

B
PYRENYLACTIN FLUORESCENCE
incubation in EGTA-buffer without Mg +/- 450 uM PIP2
assay in Ca-buffer after tenfold dilution
final [pyrenylactin] = 1.0 uM
HSS
171 +/- 9
HSS + PIP2
160 +/- 14
Control
361 +/- 10
PIP2
317 +/- 7
Time (min)

Fig. 5. (A) PIP_2 reduced the number of nucleation sites in HSS when incubated in EGTA-buffer and then assayed in buffer containing micromolar [Ca^{2+}]. A 5-μl amount of EGTA-buffer or HSS from neutrophils lysed by sonication at 3×10^8 cells/ml was incubated with 45 μl deionized–distilled water or 450 μM PIP_2 for 5 min at room temperature in EGTA-buffer. Samples were then bought to 1 ml with Ca-buffer containing 1.2 μM pyrenyl-G-actin. Steady-state fluorescence (shown next to the legend at the right of the figure) was read after overnight incubation in the dark at room temperature. (B) The removal of magnesium from the incubation buffer did not change the effects of PIP_2 in reducing the number of nucleation sites in HSS when incubated in EGTA-buffer and then assayed in buffer containing micromolar [Ca^{2+}]. A 5-μl amount of EGTA-buffer or HSS from neutrophils lysed at 2×10^8 by nitrogen cavitation was incubated with 45 μl deionized–distilled water or 450 μM PIP_2 for 5 min at room temperature in EGTA-buffer without magnesium. Samples were then bought to 1 ml with Ca-buffer containing 1.0 μM pyrenyl-G-actin. The mean fluorescence values ± the range were plotted for two independent, but identical, experiments. Steady-state fluorescence (shown next to the legend to the right of the figure) was read after overnight incubation in the dark at room temperature.
←

centrations of PIP_2 could reverse much of the effect of the HSS. As shown in Fig. 3A, the preincubation of 450 μM PIP_2 with HSS only slightly increased the nucleation rate compared to either PIP_2 or HSS alone. However, the preincubation of HSS with PIP_2 more dramatically increased the later polymerization rate compared to HSS not incubated with PIP_2, so that it approached the elongation rate of the sample with PIP_2 but without HSS. These observations are attributable to the capping activity of HSS and its inhibition by high concentrations of PIP_2.

PIP_2 micelles aggregate in buffers containing millimolar [Mg^{2+}] [22]. To test whether PIP_2 would induce more nucleation sites in HSS in the absence of magnesium, we repeated similar experiments to that described in Fig. 3A except that the incubation and assay buffers did not contain magnesium. As seen by comparing Fig. 3B to 3A, the interaction between HSS and PIP_2 was quantitatively similar in the presence or absence of magnesium. The steady-state fluorescence when PIP_2 was incubated with HSS without magnesium more closely approximated the fluorescence endpoints in the samples without HSS

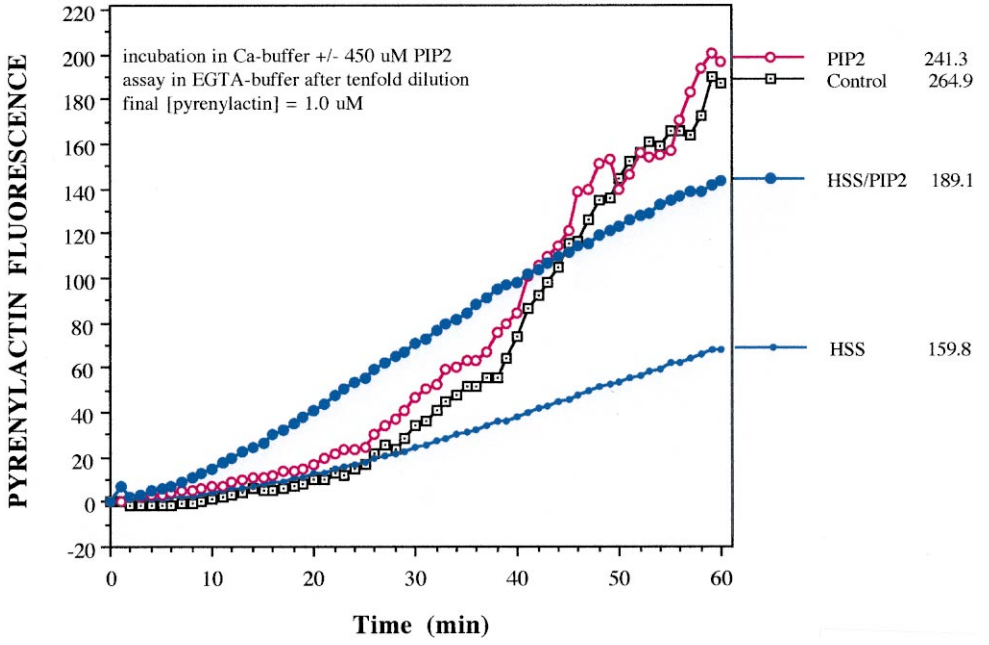


Fig. 6. PIP_2 increased the number of nucleation sites in HSS when incubated in Ca-buffer and then assayed in EGTA-buffer. A 15-μl amount of EGTA-buffer or HSS from neutrophils lysed at 2×10^8 cells/ml by nitrogen cavitation was incubated with 45 μl deionized–distilled water or 450 μM PIP_2 for 5 min at room temperature in Ca-buffer. Samples were then bought to 1 ml with EGTA-buffer containing 1 μM pyrenyl-G-actin. Steady-state fluorescence (shown next to the legend at the right of the figure) was read after overnight incubation in the dark at room temperature.

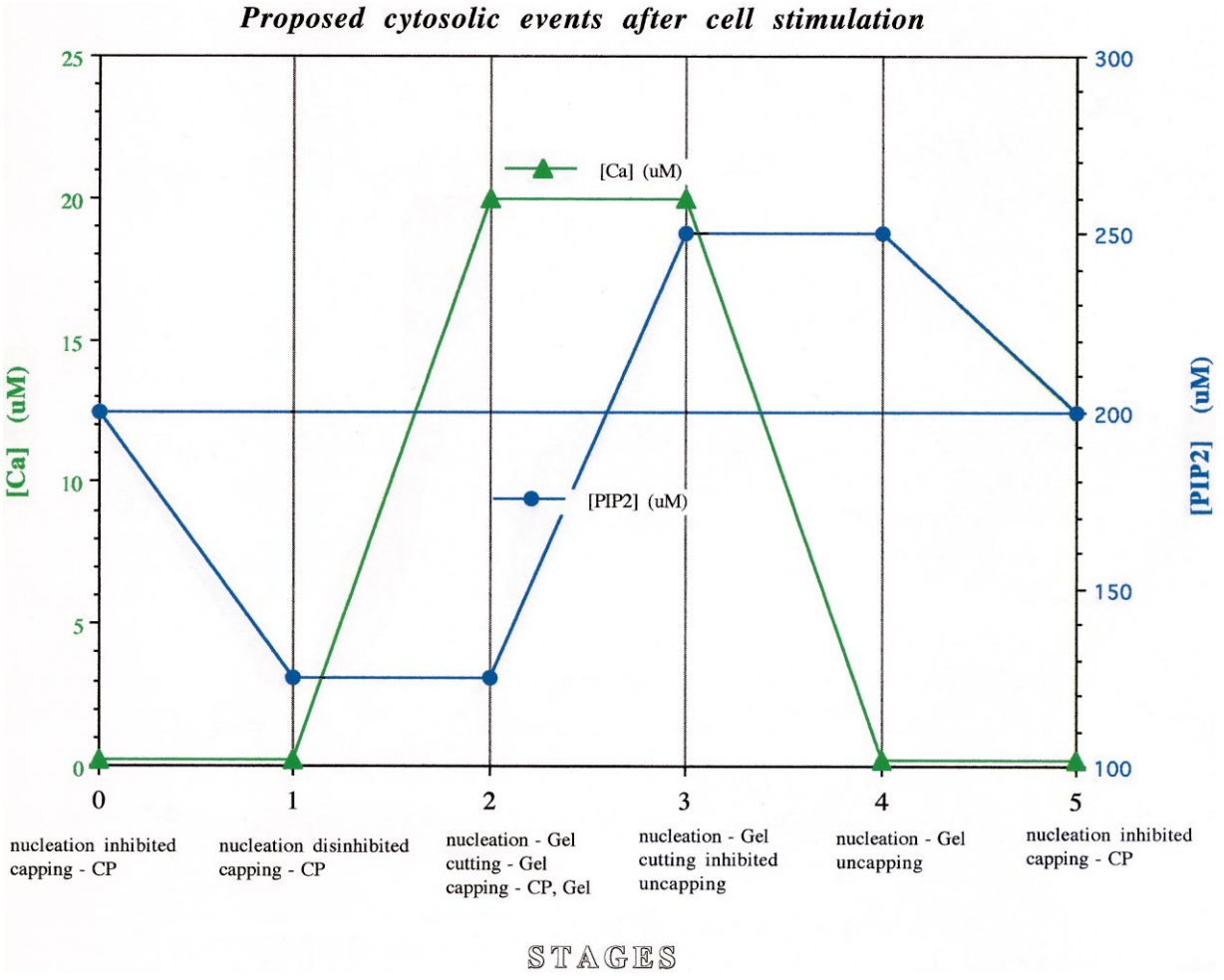


Fig. 7. A proposed sequence of events regulating cytoskeletal growth after cell stimulation. The changes in $[Ca^{2+}]$ and $[PIP_2]$ may be restricted to the cell cortex near the stimulated plasma membrane. The absolute values used for $[Ca^{2+}]$ and $[PIP_2]$ are estimates from the literature [4,25,26]. (0) In the resting cell, where the cytosolic $[Ca^{2+}]$ is submicromolar and $[PIP_2]$ is intermediate between its peak (≥ 250 μM) and nadir (≤ 150 μM) levels, most of the barbed ends on cortical actin filaments are capped by capping protein-β_2. Nucleation and cutting by gelsolin are inhibited by the resting concentrations of both Ca^{2+} and PIP_2. (1) Stimulation of the plasma membrane by certain agonists first leads to hydrolysis of PIP_2, disinhibiting gelsolin's activity and thus priming the system for nucleation and severing. (2) IP_3, generated from PIP_2 hydrolysis, increases cytosolic $[Ca^{2+}]$ and thereby activates the nucleation and severing activity of gelsolin. An increased number of shorter actin filaments capped by either gelsolin or capping protein-β_2 are quickly generated. (3) A rebound increase in $[PIP_2]$ to greater than its baseline concentration uncaps capped filament ends (and releases polymerization-competent actin from monomer-sequestering proteins). Filament severing may be inhibited. Rapid filament growth from the newly freed barbed ends ensues. (4) The subsequent decline in $[Ca^{2+}]$ in the presence of high $[PIP_2]$ prevents further filament severing and shortening while maintaining free barbed ends for elongation. Under these circumstances, neither capping protein-β_2 nor gelsolin are bound to existing filament ends, although gelsolin may continue to nucleate new filament growth. (5) Baseline conditions are restored; nucleation is again inhibited and barbed ends are recapped exclusively by capping protein-β_2.

than when magnesium was present in the buffer, perhaps attesting to the higher capacity of PIP_2 micelles in magnesium-free buffers to inhibit capping by capping protein β_2 [12].

3.3. *Preincubation of supernate with PIP_2 at submicromolar $[Ca^{2+}]$ decreased the nucleation rate when added to polymerization buffer with micromolar $[Ca^{2+}]$*

The ability of PIP_2 to affect the late polymeriza-

Table 1
Reduction in number of calcium-inducible nucleation sites in high speed supernates after preincubation with different phosphoinositols

Incubation condition (all at submicromolar [Ca^{2+}])		Initial polymerization rate HSS+phosphoinositol	Initial polymerization rate HSS alone	Relative number of nuclei (HSS+phosphoinositol)/ (HSS alone)
PIP_2	With Mg	15.7	23.4	0.67
PIP_2	Without Mg	10.4	18.7	0.56
PIP	With Mg	20.0	22.4	0.89
PI	With Mg	28.8	30.8	1.07

Initial polymerization rates were measured from the increase in pyrenylactin fluorescence during the first 2 min immediately following dilution of 5 μl HSS (from neutrophils lysed at 2×10^8 cells/ml by nitrogen cavitation), which had been incubated at room temperature with or without 45 μg of sonicated phosphoinositol in 100 μl EGTA-buffer in the presence or absence of magnesium for 5 min, into 1 ml Ca-buffer containing 0.8 μM pryrenyl-G-actin. The relative number of nuclei were calculated as the ratio of the initial polymerization rate for the sample with relative to the corresponding sample without phosphoinositol. HSS, high speed supernate (of neutrophil extract); PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP, phosphatidylinositol.

tion rate when incubated and assayed at micromolar [Ca^{2+}] was negligible, regardless of the presence of HSS (Fig. 4). However, PIP_2 did slow nucleation by HSS to a minor degree. The same results were observed when 1 μM exogenous actin was added during the preincubation.

Compared to the calcium-containing buffer, incubation of PIP_2 and HSS in EGTA-containing buffer resulted in a larger decrease in the initial polymerization rate when subsequently assayed at micromolar [Ca^{2+}] (Fig. 5A). Some inhibition of nucleation by preincubating HSS and PIP_2 was expected since PIP_2 decreases the nucleating capacity of purified gelsolin under these conditions. Similar effects were found when the incubation buffer did not contain magnesium (Fig. 5B). The inhibition of nucleation was less with PIP than PIP_2; no consistent inhibition was demonstrable with PI (Table 1).

3.4. *Preincubation of supernate with PIP_2 at micromolar [Ca^{2+}] preserved some nucleation sites when diluted into EGTA-containing polymerization buffer without calcium*

In contrast to the above results, the incubation of HSS with PIP_2 at micromolar [Ca^{2+}], followed by diluting 10-fold into an EGTA-containing assay buffer, yielded more nucleation sites than similarly treated HSS not exposed to PIP_2 (Fig. 6). Stated differently, it appeared that the presence of high concentrations of PIP_2 during the preincubation maintained nucleation sites in the HSS after chelation of calcium with EGTA. In contrast, the preincubation of PIP_2 and HSS in polymerization buffer containing micromolar [Ca^{2+}] and 1 μM actin decreased the nucleation rate upon 10-fold dilution into an EGTA-containing assay buffer, consistent with the findings depicted in Fig. 4. These data indicate that, although less nuclei had been created in HSS incubated with as opposed to without PIP_2 at micromolar [Ca^{2+}], more of these nucleation sites remained active after the [Ca^{2+}] had been lowered with EGTA. Since elongation from these nuclei could only occur from the pointed ends, we confirmed that cytochalasin B would not be expected to slow polymerization under these conditions. Although our other results with HSS assayed in Ca^{2+}-buffer could be reproduced when purified gelsolin was substituted for HSS, the sequential exposure of purified gelsolin to Ca^{2+}-buffer with PIP_2 followed by EGTA-buffer did not lead to more retained nucleation sites than in the absence of PIP_2.

4. Discussion

At submicromolar [Ca^{2+}], high concentrations of PIP_2 induce only a small increase in nucleation sites in HSS, but can prevent capping by HSS of dilute neutrophil lysates. As previously demonstrated using exogenous spectrin-actin seeds as nuclei, PIP_2 inhibits the gradual slowing of the elongation rate caused by HSS through inhibition of capping protein-β_2 [14,20]. The addition of micromolar [Ca^{2+}] to HSS activates the nucleating activity of gelsolin, leading to

'explosive' polymerization. The final extent of pyrenylactin polymerization is lower with HSS both in the presence and absence of micromolar [Ca^{2+}], implying that the large majority of barbed filament ends are capped (albeit by different cappers) at steady state under either buffer condition. Exposure of HSS to PIP_2 decreases the number of calcium-inducible nucleation sites, especially if preincubated at submicromolar [Ca^{2+}]. Preincubation of HSS with PIP_2 at micromolar [Ca^{2+}] paradoxically maintains some active nucleation sites after the [Ca^{2+}] has been lowered to submicromolar levels.

The nucleating effect of gelsolin requires activation by calcium. PIP_2 also modulates the binding of gelsolin to actin monomers and filaments. Our data suggest that the interactive effects of these two regulatory mechanisms on gelsolin may not be simply additive. Interestingly, Janmey et al. [11] had earlier reported that gelsolin could quickly be removed from the barbed end by EGTA only if exposed to PIP_2 *before* capping. Others have more recently demonstrated that the affinity of gelsolin for PIP_2 is increased by calcium [23]. We theorize that both the on- and off-rates for the binding of Ca^{2+} to gelsolin are slowed by PIP_2. Our data are consistent with the hypotheses that the binding of PIP_2 to gelsolin can interfere with the subsequent binding of Ca^{2+}, while the binding of Ca^{2+} before PIP_2 may inhibit the release of Ca^{2+} from gelsolin *in HSS* when [Ca^{2+}] is later lowered by EGTA. Thus, compared to similar conditions in the absence of PIP_2, exposure of gelsolin in HSS to PIP_2 at submicromolar [Ca^{2+}], followed by an increase in the [Ca^{2+}] to the micromolar range, reduces nucleation sites, whereas the coincidental exposure of gelsolin to micromolar [Ca^{2+}] and PIP_2 maintains more nucleating sites after [Ca^{2+}] is lowered. Since we could not reproduce this latter effect with purified gelsolin, a cofactor in HSS might be required in this process.

At first glance, our data may appear to contradict recent work by Zigmond and colleagues [9]. These investigators showed that PIP_2, even at concentrations as high as 400 μM, did not affect the amount of endogenous actin in neutrophil HSS that could be induced to polymerize by GTPγS in a EGTA-containing polymerization buffer. Under similar assay conditions in our experiments, PIP_2 only minimally increased the nucleation rate and extent of pyrenyl-actin polymerization when added to HSS in the absence of micromolar [Ca^{2+}]; its major effect was to decrease the gradual slowing of filament elongation by HSS. We also demonstrated that the nucleation of pyrenylactin polymerization in Ca-buffer could be increased or decreased by preincubating HSS with PIP_2 at micromolar or submicromolar [Ca^{2+}], respectively, without significantly affecting the final extent of polymerization. One plausible 'unifying' hypothesis for both sets of data is that the key role of PIP_2 is to modulate the rate, not the extent, of actin polymerization.

4.1. Limitations of studying high speed supernate of dilute neutrophil lysates

The study of HSS can hopefully provide a cell-free system for investigating the interactive effects of actin regulatory proteins in a complex milieu simulating the cytosolic conditions [9]. Cells are unavoidably perturbed by lysis and dilution, and potentially intertwined systems may be disrupted and partially spun down by our protocols for producing HSS. It is certainly possible that signaling pathways in dilute HSS 'spiked' with pyrenyl-G-actin might not reflect in vivo events very closely. Nevertheless, examining the regulation of actin filament growth by HSS exposed to intracellular messengers will likely yield some novel insights beyond those already gleaned from the study of highly purified components.

Another confounding problem in studying cell lysates is that only composite effects of the specimens can be measured. Some degree of compartmentalization may be restored by fractionating the lysate, as we have done in preparing HSS. Since the fine regulation of cytoskeletal assembly likely depends in large part on the precise localization and concentration of both actin binding proteins and intracellular messengers, speculation about the function of in vivo systems derived from the work reported here can only be tentatively advanced in the face of these methodological limitations.

4.2. Further cautions and qualifications

The nucleation rate of pyrenylactin polymerization in Ca-buffer was affected by the [Ca^{2+}] and [PIP_2] preincubated with HSS. We have heretofore de-

scribed these effects in terms of changes in the number of competent nucleation sites in HSS under different conditions. An equally satisfactory explanation for our observations would be a change in the efficiency of nucleation without necessarily changing the number of sites. Thus, gelsolin may be a more or less effective nucleating protein depending on the conditions of the preincubation to which the HSS was subjected.

The effects of the high [PIP_2] used in our experiments might be at least partially mediated through PIP_2-induced reductions in the concentrations of key ions, such as K^+, Mg^{2+} and Ca^{2+}. Given the relatively low [PIP_2] in the assay (as opposed to the incubation) buffer and the minimal effect of removing magnesium from the assay buffer, potential changes in the free [K^+] or [Mg^{2+}] caused by PIP_2 seem unlikely explanations for any of our observations. Moreover, the 45 μM PIP_2 present in our assays would not be expected to lower the 200 μM calcium in our Ca-buffer to submicromolar [Ca^{2+}].

Our most surprising finding was that HSS sequentially exposed to *Ca-* and then *EGTA-buffer* retained more nucleation sites if PIP_2 was present in the Ca-buffer. We could not demonstrate this effect using purified gelsolin instead of HSS. Moreover, although we 'successfully' reproduced this result, there were many 'negative' experiments using different HSS preparations. Whether this variability represents denaturing of a critical cofactor in HSS is presently unknown. PIP_2 may inhibit the binding of gelsolin to monomeric actin in the HSS during the preincubation in *Ca-assay buffer*. If free gelsolin were a more efficient actin nucleator than gelsolin–actin complexes in *EGTA-buffer*, this mechanism could account for some of the observed differences in nucleation sites. Until we understand the determinants of this paradoxical observation, we do not want to unduly emphasize its physiological significance.

4.3. *Biological relevance: extrapolation and speculation*

The stimulated neutrophil must simultaneously promote and prevent actin polymerization in bordering cytoskeletal domains at different times. The kinetics of filament assembly is a function not only of the concentration of various intracellular messengers (e.g., Ca^{2+} and PIP_2), but also of the location and sequence of their generation. The following undoubtedly oversimplified model attempts to integrate our data into current concepts of cytoskeletal regulation [4,7,9,12,24] (Fig. 7).

The resting neutrophil prevents actin assembly by multiple distinct mechanisms, including capping the barbed ends of preformed filaments and inhibiting nucleation of new filaments. This latter task is accomplished by inactivating nucleating proteins as well as decreasing the polymerization-competent G-actin concentration to limit spontaneous nucleation. Gelsolin acts as a potent nucleator of actin polymerization exclusively in the presence of micromolar [Ca^{2+}]. In addition, PIP_2 can inhibit nucleation by gelsolin when mixed together before [Ca^{2+}] is raised to micromolar levels. We theorize that PIP_2 helps protect against gelsolin-nucleated filament growth if the [Ca^2] is increased but [PIP_2] is not simultaneously lowered. A fall in resting [PIP_2] disinhibits gelsolin's nucleating activity and primes the system for Ca^{2+}-initiated nucleation. The subsequent rebound in [PIP_2] may not fully reverse the nucleating activity of gelsolin, but serves primarily to uncap barbed ends blocked by capping protein-β_2. Once the [Ca^{2+}] is returned to submicromolar levels, high [PIP_2] will also uncap gelsolin-capped filaments. This series of events leads to an increase in free filament ends by nucleating new filament growth, cutting pre-existing filaments and ultimately uncapping barbed ends so that a new set of more numerous but shorter filaments can rapidly elongate.

Acknowledgements

My deep appreciation and gratitude are extended to Susan Alteri Ruickholt and Michael J. DiNubile for their support, help and encouragement throughout this project. I also thank Yolanda Smith, Jennifer Viner and Diana DeCillis for their expert technical and secretarial contributions, respectively. Finally, I am once again indebted to Dr. Sally Zigmond in the Department of Biology at the University of Pennsylvania (NIH Grant AI19883) for her continuing support and invaluable advice. This work was supported by Cooper Faculty Grant SPF 2821-94.

References

[1] M. Fechheimer, S.H. Zigmond, Focusing on unpolymerized actin, J. Cell Biol. 123 (1993) 1–5.

[2] L.U. Cassimeris, H. McNeill, S.H. Zigmond, Chemoattractant-stimulated polymorphonuclear leukocytes contain two populations of actin filaments that differ in their spatial distributions and relative stabilities, J. Cell Biol. 110 (1990) 1067–1075.

[3] M.L. Cano, D.A. Lauffenburger, S.H. Zigmond, Kinetic analysis of F-actin depolymerization in polymorphonuclear leukocyte lysates indicates that chemoattractant stimulation increases actin filament number without altering the filament length distribution, J. Cell Biol. 115 (1991) 677–687.

[4] J.H. Hartwig, G.M. Bokoch, C.L. Carpenter, P.A. Janmey, L.A. Taylor, A. Toker, T.P. Stossel, Thrombin receptor ligation and activated rac uncap actin filament barbed ends through phosphoinositide synthesis in permeabilized human platelets, Cell 82 (1995) 643–653.

[5] L.G. Tilney, P. Connelly, Smith, G.M. Guild, F-Actin bundles in *Drosophila* bristles are assembled from modules composed of short filaments, J. Cell Biol. 135 (1996) 1291–1308.

[6] J.H. Hartwig, Mechanisms of actin rearrangements mediating platelet activation, J. Cell Biol. 118 (1992) 1421–1442.

[7] K. Barkalow, W. Witke, D.J. Kwiatkowski, J.H. Hartwig, Coordinated regulation of platelet actin filament barbed ends by gelsolin and capping protein, J. Cell Biol. 134 (1996) 389–399.

[8] M. Tardif, S. Huang, T. Redmond, D. Safer, M. Pring, S.H. Zigmond, Actin polymerization induced by GTPγS in permeabilized neutrophils is induced and maintained by free barbed ends, J. Biol. Chem. 270, (47) (1995) 28075–28083.

[9] S.H. Zigmond, M. Joyce, J. Borleis, G.M. Bokoch, P.N. Devreotes, Regulation of actin polymerization in cell-free systems by GTPγS and cdc42, J. Cell Biol. 138, (2) (1997) 363–374.

[10] P.A. Janmey, T.P. Stossel, Modulation of gelsolin function by phosphatidylinositol 4,5-bisphosphate, Nature 325 (1987) 362–364.

[11] P.A. Janmey, K. Iida, H.L. Yin, T.P. Stossel, Polyphosphoinositide micelles and polyphosphoinositide-containing vesicles dissociate endogenous gelsolin-actin complexes and promote actin assembly from the fast-growing end of actin filaments blocked by gelsolin, J. Biol. Chem. 262 (1987) 12228–12236.

[12] D.A. Schafer, P.B. Jennings, J.A. Cooper, Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides, J. Cell Biol. 135, (1) (1996) 169–179.

[13] L.J. Pike, L. Casey, Localization and turnover of phosphatidylinositol 4,5-bisphosphate in caveolin-enriched membrane domains, J. Biol. Chem. 271 (1996) 26453–26456.

[14] M.J. DiNubile, S. Huang, High concentrations of phosphatidylinositol-4,5-bisphosphate may promote actin filament growth by three potential mechanisms: inhibiting capping by neutrophil lysates, severing actin filaments, and removing capping protein-β_2 from barbed ends, Biochim. Biophys. Acta 1358 (1997) 261–278.

[15] S.J. Sullivan, S.H. Zigmond, Chemotactic peptide receptor modulation in polymorphonuclear leukocytes, J. Cell Biol. 85 (1980) 703–711.

[16] M.S. Klempner, R.B. Mikkelsen, D.H. Corfman, J. Andre-Schwartz, Neutrophils plasma membranes I. High-yield purification of human neutrophil plasma membrane vesicles by nitrogen cavitation and differential centrifugation, J. Cell Biol. 86 (1980) 21–28.

[17] J.A. Spudich, S. Watt, The regulation of rabbit skeletal muscle contraction. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin, J. Biol. Chem. 246 (1971) 4866–4871.

[18] J.M. Murray, A. Weber, M.K. Knox, Myosin subfragment I binding to relaxed actin filaments and the steric model of relaxation, J. Biol. Chem. 20 (1981) 641–649.

[19] J.A. Cooper, Effects of cytochalasin and phalloidin on actin, J. Cell Biol. 105 (1987) 1473–1478.

[20] M.J. DiNubile, L. Cassimeris, M. Joyce, S.H. Zigmond, Actin filament barbed-end capping activity in neutrophil lysates: the role of capping protein-β_2, Mol. Biol. Cell. 6 (1995) 1659–1671.

[21] S. MacLean-Fletcher, T.D. Pollard, Mechanism of action of cytochalasin B on actin, Cell 20 (1980) 329–341.

[22] P.A. Janmey, T.P. Stossel, Gelsolin-polyphosphoinositide interaction: full expression of gelsolin-inhibiting function by polyphosphoinositides in vesicular form and inactivation by dilution, aggregation, or masking of the inositol head group, J. Biol. Chem. 264 (1989) 4825–4831.

[23] K. Lin, E. Wenegieme, P. Lu, C. Chen, H.L. Yin, Gelsolin binding to phosphatidylinositol 4,5 bisphosphate is modulated by calcium and pH, J. Biol. Chem. 272 (1997) 20443–20450.

[24] M.F. Carlier, D. Pantaloni, Control of actin dynamics in cell motility, J. Mol. Biol. 299 (1997) 459–467.

[25] B.W. Agranoff, P. Murthy, E.B. Seguin, Thrombin-induced phosphodiesteratic cleavage of phosphatidylinositol bisphosphate in human platelets, J. Biol. Chem. 258 (1983) 2076–2078.

[26] P. Grondin, M. Plantavid, C. Sultan, M. Breton, G. Mauco, H. Chap, Interaction of $pp60^{c-src}$, phospholipase C, inositol-lipid, and diacylglycerol kinases with the cytoskeleton of thrombin-stimulated platelet, J. Biol. Chem. 266 (1991) 15705–15709.